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ANALYSIS OF THE HUMAN T CELL RESPONSE TO HTLV-III

Annual Report

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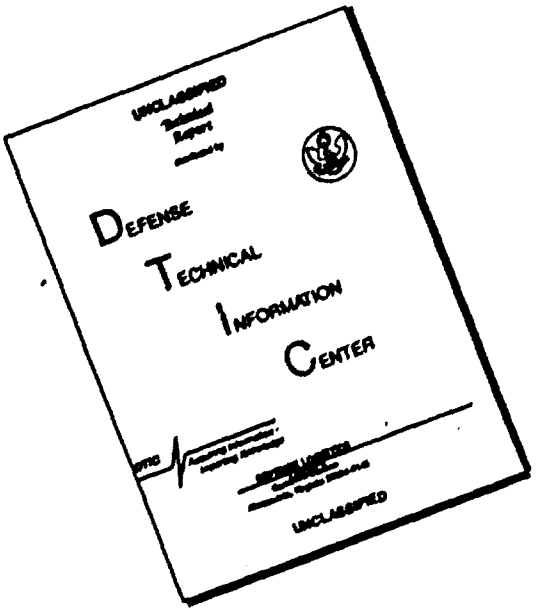

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Acquired Immune Deficiency Syndrome is marked by a collapse in immune responses, particularly those mediated by thymus-derived (T) lymphocytes. Nevertheless, the presence of antibodies to the AIDS virus (HIV) indicates the presence of virus specific T cells. Over the last year we have defined cell tumors and populations which are much less susceptible to virus mediated killing. We are currently investigating how cells differ in their sensitivity to virus and how we can utilize this information therapeutically.

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FOREWORD

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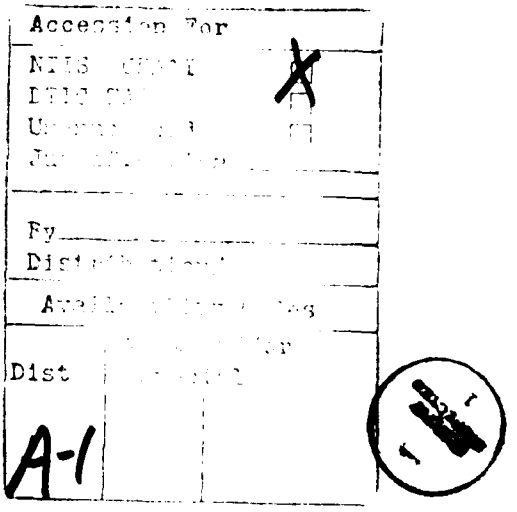

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I. STATEMENT OF THE PROBLEM:

Although patients with HIV infection have depressed T cell function, the fact that they produce antibodies to HIV proteins (1) and even have lymphocytes capable of lysing infected target cells (2) indicates the presence of an immune response to this virus. The purpose of this contract is to devise methods to characterize that T cell response in a variety of patient populations. Once the T cell response has been defined, it should be possible to attempt to augment any response which could be effective at the elimination of virus as well as potentially dampening ineffective responses, eliminating unwanted responses. We are concerned with two major issues with regard to the interaction of T cells and HIV: 1) what make some T cells susceptible to virus?; and 2) why are some CD4 cells apparently virus resistant?

II. BACKGROUND AND REVIEW OF APPROPRIATE LITERATURE

In formulating our hypothesis, we relied on the fact that patients with HIV infection have a good antibody response to the viral proteins (1) and the fact that in other human retroviruses (e.g., HTLV-1) cytolytic T cells can be demonstrated (3, 4). Recently Walker and others have demonstrated the presence of lymphocytes with the capacity to lyse infected target cells in the blood of patients with HIV (2). Although previous studies of virus specific T cells had concentrated on surface glycoproteins, recent data indicates that internal proteins may be important in stimulating T cells (5). For these reasons, we are utilizing a variety of proteins as stimuli. The recent use of peptides rather than whole proteins as stimuli for target for CTL (6) has broadened our horizons of potential antigens which can be evaluated.

Recent publications document that HIV-1 specific T cell clones can be derived from AIDS patients (7). However, despite the presence of such clones, the disease progresses. Therefore, either these clones are not effective at eliminating the virus or they themselves are sensitive to the virus. Thus, we are currently investigating ways by which clones which are not susceptible to infection may be induced. In the last few years, a group of $CD4^-$ T cells have been described. These cells which are like conventional $CD4^+$ or $CD8^+$ T cells are $CD3^+$ and have a receptor characterized by a two chain structure. However, instead of having the $\alpha\beta$ dimer found in 90% of T cells, this subset has an alternative structure referred to as the $\gamma\delta$ receptor (8). The importance of such cells has not been defined.

It is conceivable that CD4- T cells will be resistant to infection with HIV-1 (since HIV-1 binds to the target cells by utilizing CD4 as a receptor). While the presence of CD4 seems to be sufficient for infection to occur in certain circumstances, we have found situations in both human and murine cells where the presence of CD4 is not sufficient for HIV-1 infection (see Results section). This has led us to investigate differences between CD4 cells which might result in some being resistant to infection.

III. RATIONALE USED IN CURRENT STUDY

Our eventual goal (as stated in the contract proposal) will be to augment protective immunity. In order to obtain such reagents, however, it is necessary to find T cell clones which will: 1) protect against virus; and 2) resist virus infection. To do this, we have had to develop methods to 1) support the growth of T cells from HIV positive patients and 2) define what antigens will be stimulated.

In addition to defining the presence of virus specific T cell clones as others have done (7), we are approaching the question of how a T cell may be resistant to viral infection. We are doing this by utilizing $CD4^-$ tumor cells (both human and mouse) and then transfecting them with CD4.

IV. EXPERIMENTAL METHODS

In order to support the growth of lymphocytes from HIV positive patients, we have used the following protocol:

Isolation of Mononuclear Peripheral Blood Cells and Proliferation Assays

Human mononuclear peripheral blood cells (PBL) were isolated under sterile conditions from 60-100 ml heparinized peripheral blood by ficoll-hypaque density gradient centrifugation. Thirty ml aliquots of blood were layered over 15 ml of lymphocyte separation (LSM) medium (Litton Bionetics, Kensington, MD). After centrifugation, cells at the interface were collected, and washed twice in a culture medium consisting of RPMI 1640 medium (Gibco, Grand Island, New York) supplemented with 5% human AB serum (MA Bioproducts, Walkersville, MD), 2mM glutamine (Gibco), and 100 U/ml penicillin-streptomycin (Gibco).

Generation of $CD4^-$, $CD8^-$ ($\gamma\delta^+$) T cells

PBMCs from blood drawn from a healthy PPD-reactive individual were separated by sodium diatrizoate/Ficoll centrifugation and then cultured at 2×10^6 ml^{-1} in complete medium (RPMI 1640 plus 15% autologous serum plus 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin) together with 10 μg ml^{-1} PPD (Statens Seruminstitut, Copenhagen) in a 25-cm^3 flask (Costar) and incubated at 37°C in a 5% CO_2/95% air. On day 6, viable cells were separated by density-gradient centrifugation and recultured with fresh autologous irradiated (4,000 rad) PBMC feeders and PPD. On day 9, viable cells were recovered and $CD4^-$ and $CD8^+$ cells depleted. Briefly, the cells were incubated with supernatants from OKT4 and OKT8 hybridomas for 30 min at 4°C and then washed and further incubated with goat-anti-mouse-antibody coated latex-polymer beads with a magnetic core (Advanced Magnetics) at 37°C, on a gyratory shaker for 20 min. Subsequently, $CD4^+$ and $CD8^+$ cells bound to the beads were removed by a magnet (BioMag Separator, Advanced Magnetics). The remaining cells were washed and cultured with irradiated autologous feeders (1×10^6 ml^{-1}), PPD, and 10% T-cell growth-factor-containing media (Lymphocult, Biotest AG, Frankfurt). This cycle was repeated every 7 days and the expanded cells were depleted of $CD4^+$ and $CD8^+$ cells repeatedly, together with an anti-$\alpha\beta$ (BMA 031) antibody for depletion of $\alpha\beta$ lymphocytes. Phenotypic marker analysis was determined by standard double-color FACS. The monoclonal antibodies used were anti-Leu-4-PE (Becton) for the

CD3 marker, BMA 031 for αβ, and TCR δ1 for γδ. For the unlabelled antibodies, a second layer of labelled goat-anti-mouse-FITC (Becton) was applied before FACS analysis. Controls were run with isotype-matched antibodies.

Production and Infection of CD4 Expressing Human Tumor Cells

HSB2, a $CD4^-$ human T cell tumor, was cocultured with the retroviral producer lines MNST4 DAMP (9) and MNCT4PI DAMP in order to confer expression of CD4 and CD4PI, respectively. The protein encoded by this hybrid gene has authentic CD4 sequence up to amino acid 371 and, following a five amino acid insertion shown in single letter code, the authentic sequence of the GPI-anchored form of LFA-3 from amino acid 210. The hybrid gene consists of CD4 cDNA sequence through the Fnu4HI site at 1257 (10) where a BamHI linker was inserted. It then continues, to the end of LFA-3, through a BamHI linker inserted in the NspBII site at 636 (11). These producer lines are obtained by transfection of the amphotropic helper line DAMP with defective proviral vectors carrying neomycin resistance and the gene in question, as has been previously described (9, 12, 13). The HSB2 infectants were selected in the antibiotic G418 and screened for expression of CD4(PI) by immunofluorescent staining with OKT4. $CD4^+$ lines chosen for initial experiments were subsequently cloned by limiting dilution. Cells were stained with OKT4 hybridoma supernatant and FITC (fluorescein isothiocyanate) goat anti-mouse IgG $(Fab')_2$ (FITC GAM) (Tago) or FITC GAM alone and fixed in 1% paraformaldehyde. The clones derived after transfection with CD4PI (e.g., P17.13) had a CD4 molecule whose surface expression was diminished by incubating with phosphatidyl inositol specific phospholipase (PIPLC). Other clones derived from conventional CD4 constructs (e.g., CD410) were membrane anchored and therefore expression of CD4 was not susceptible to cleavage by PIPLC.

Production and Infection of CD4 Expressing Murine Tumor Cells

cDNAs encoding the human CD4 protein were introduced into two murine T cell hybridomas, By155.16 and 5D5.63 by cocultivating the hybridomas with the amphotropic producer line, MNST4 DAMP (9). By155.16 is the product of a C57BL/6 splenic T cell fused with the thymoma BW5147. 5D5.63 is an autoreactive T cell hybridoma generated by fusing cultured lymph node cells from an autoimmune MRL/Mp-lpr/lpr mouse with BW5147. Expression of human CD4 proteins was demonstrated in all cells by staining with monoclonal anti-OKT4, OKT4a, and Leu 3a antibodies.

RESULTS

I. CD4 expressing T cell clones may not be infectable with HIV-1

A. The ability to infect a human T cell clone is dependent not only on its ability to express CD4 but also on its ability to internalize the CD4 molecule.

Monoclonal populations of the HSB tumor transfected with CD4 we restained for expression of CD4. CD4 expressing cells were divided into high expressors (clone M23 is shown in Figure 1) or low expressors (clone P17.13 is shown in Figure 1).

When infected with HIV-1, the "low" expressing clones more easily infected with HIV-1 with expression of viral proteins occurring within a few days (Figure 2).

High expressing clones were not infectable even with extremely high titers of virus. In order to investigate whether the level of expression of CD4 was responsible for the lack of infection, cells were preincubated with a non-blocking monoclonal antibody to CD4 ($OKT4_c$) and goat anti-mouse antibody. Such preincubated cells were capable of infection with HIV. While 10.28 and M23 could not be infected without "modulation of CD4," both expressed p24 antigens within six days of infection when the incubation was carried out with CD4 antibodies and a goat anti-mouse second antibody.

B. Mouse T cell clones which express human CD4 are only infectable if the human CD4 is capable of modulation by the murine cell.

The parental mouse cell line 5D5.63, human CD4 bearing murine T cell hybridomas (63.CD4.33 and 16.CD4.9), and a human CD4+ T cell lines H9 were exposed to HIV-1 *in vitro*. P24 production was quantitated at different times after infection using a p24 specific ELISA assay (Abbott). Only 63.CD4.33 and the human cell lines H9 were infected with HIV-1 (Figure 3). Infection of 63.CD4.33 and H9 was inhibited by incubating cells with OKT4a antibody but not by OKT4 antibody indicating that the virus bound to the same epitope on the murine cells as on human T cells (results not shown). Culture supernatants from infected 63.CD4.33 will infect human H9 cell lines, indicating infectious viral particles were produced by the murine cell lines (results not shown).

II. Antigen specific CD4 negative human T cell clones can be derived from peripheral blood

In a study aimed at elucidating the participation of $TCR_{\gamma\delta}^+$ T cells in the immune response to antigen, we cultured peripheral blood mononuclear cells (PMC) from an immune individual with PPD for six days and analyzed the change in the percentage of TCR $_{\gamma\delta}^+$ lymphocytes by flow cytometry after staining with antibody directed at the δ chain of the TCR $_{\gamma\delta}$ heterodimer. The results of such studies revealed a fourfold increase from the baseline of 4% $TCR_{\gamma\delta}^+$ of the $CD3^+$ T cells in the unstimulated PBMC to 16% after stimulation with PPD (data not shown). This expansion of $CD3^+TCR_{\gamma\delta}^+$ lymphocytes may indicate either an antigen specific stimulation of these lymphocytes, or a non-specific bystander and passive expansion of these cells in response to growth factors produced by the predominant $TCR_{\alpha\beta}^+$ cells. We therefore designed experiments to find out if there is an antigen specific expansion of $TCR_{\gamma\delta}^+$ T cells.

In order to isolate antigen reactive $TCR_{\gamma\delta}^+$ T cells, we raised a PPD reactive line by stimulating PBMC with PPD and by restimulation with antigen and fresh feeders for an additional three day period. Subsequently, we enriched for $TCR_{\gamma\delta}^+$ cells by negatively depleting $CD4^+$ and $CD8^+$ lymphocytes by incubating the line with anti-CD4 and anti-CD8 monoclonal antibodies and removing the $CD4^+$ and $CD8^+$ cells with anti-mouse immunoglobulin coated latex beads. Such CD4 and CD8 depleted cells were then expanded by incubating with fresh antigen presenting cells, PPD and T cell growth factor containing media. Further serial depletion of this line with anti-CD4 and anti-CD8 antibody followed by an anti-$TCR_{\alpha\beta}$ antibody resulted in a homogeneous and stable $TCR_{\gamma\delta}^+/_{\alpha\beta}^-$

$CD3^+CD4^-CD8^-$ line (GD line) as revealed by double staining with anti-CD3 antibody and the appropriate anti-TCR antibodies or double staining with anti-CD4 and anti-CD8 antibodies (Fig. 4). Furthermore, immunoprecipitation of ^{125}I-labelled GD cells with anti-TCRγ1 and $TCR_{c\gamma}$ antibody revealed a 40 kDa TCRδ chain and a 55kDa TCRγ chain, respectively (Fig. 5). The δ subunit showed a marked shift in mobility under reducing and non-reducing conditions consistent with previous reports for $TCR_{\gamma\delta}^+$ T cells. These cells were also shown by flow cytometry to express CD2 (sheep red blood cell receptor), UCHL-1, the activation antigens HLA-DR and the IL-2 receptor, but lacked CD45R, and CD16 (Fcγ receptor and a natural killer cell marker) antigens. The purity of the line was functionally confirmed by incubating the line with monoclonal antibodies directed at components of the TCR complex, namely, CD3, and δ chain and the αβ complex in the presence of plastic adherent APC. The results of such studies (data not shown) revealed the line to proliferate in response to either anti-TCRδ1 or anti-CD3 but not to anti-$TCR_{\alpha\beta}$ antibodies providing further evidence that the GD line is composed only of $TCR_{\gamma\delta}$+ cells.

The specific functional capability of the GD line was tested by a proliferative assay against PPD and an irrelevant antigen. For such studies, GD cells were cultured together with autologous APC in the presence of either PPD, formalin inactivated candida antigen, or media alone. The results of such experiments (Fig. 6a) showed a marked proliferative response to PPD but essentially no response to candida antigen, reflecting the specificity of these $TCR_{\gamma\delta}^+$ lymphocytes to PPD. These findings, therefore, provide evidence that $TCR_{\gamma\delta}^+$ T cells do indeed recognize antigen specifically as do $TCR_{\alpha\beta}^+$ lymphocytes. Since PPD is a crude culture filtrate of Mycobacterium bovis strain BCG and contains several tuberculoproteins, we obtained a well-characterized and purified mycobacterial antigen in order to better assess antigen specificity. We therefore tested the response of the GD line to purified recombinant 65 kD HSP cloned from BCG/6. The results of such studies (Fig. 6b) demonstrated the line to have a vigorous response to this antigen, indicating the 65 kDa mycobacterial HSP to be an important inducer of $TCR_{\gamma\delta}^+$ T cells.

DISCUSSION AND CONCLUSIONS

The long term goal of these studies is to find ways of amplifying the human T cell response to HIV-1 in such a manner that it is useful in protecting against the development of AIDS.

In approaching this problem, two obvious problems present themselves. The first problem relates to the fact that patients with HIV-1 infection have poor T cell responses to all specific antigens. We have approached this problem by using an immunomodulator: IFN-γ (which is also an anti-viral agent) to enhance antigen specific immune responses. These studies (concluded in the first year of the contract) are described in our publication "Recombinant Human IFN-γ enhances *in vitro* activation of lymphocytes isolated from patients with AIDS" (Infection and Immunity, in press).

The problem with which we have dealt in this portion of the contract concerns the development of clones and their resistance to the lytic effects of the HIV-1 virus. We have used two approaches to investigate this problem.

Taking advantage of the fact that HIV-1 infects cells via CD4, we have established antigen specific T cells which are CD4-. We have found that these cells are $CD3^+$ and bear the γδ T cell receptor. Whether such cells (which might be anticipated to be resistant to

HIV-1) will be susceptible to the virus is currently under study. If they are resistant, we will concentrate on isolating such $\gamma\delta$ clones (in preference to $\alpha\beta$ clones) because of their ability to withstand viral infection.

In a murine model of infection, we have demonstrated that some virus specific T cell clones may be resistant to lysis by a virus despite the presence of surface receptors (Horn et al, J. Virology, in press). It will be essential in future work to determine whether we can isolate such clones from patients with HIV-1. Such studies will be pursued in the next year of the proposal.

Another strategy to develop resistant cells is to alter the CD4 molecule in a manner which prevents HIV-1 infection. We have demonstrated that the CD4 molecule does not require a membrane anchor in order to serve as a virus receptor (Diamond DL, Finberg R, Chaudhuri S, Sleckman BP, and Burakoff SJ, HIV infection is efficiently mediated by a glycolipid anchored form of CD4, submitted to Science).

The converse of this statement is shown in the other experiment we have performed using transfected tumors. In these experiments, a high level of CD4 expression negatively affects the ability of HIV-1 to infect the cell. A reduction in CD4 expressing (activated by antibody induced modulation) resulted in infection. These data and the results obtained with the murine clones both suggest that internalization of a CD4-HIV-1 complex may be essential for HIV-1 infection.

A cell line which produces a high level of CD4 but does not internalize the CD4-virus complex is not susceptible to infection. These data suggest that immunological or pharmacologic manipulations which increase CD4 expression and decrease CD4 internalization may decrease HIV infection of T cells. Since the major reservoir of the virus in AIDS patients appears to be circulating $CD4^+$ T cells, these manipulations may be of therapeutic importance.

FIGURE LEGENDS

Figure 1. CD4 staining of high expressor (CD4M) and low expressor (P17.13) transfectants of HSB (parent line).

Figure 2. Low expressor HSB transfectants are susceptible to HIV-1 infection.

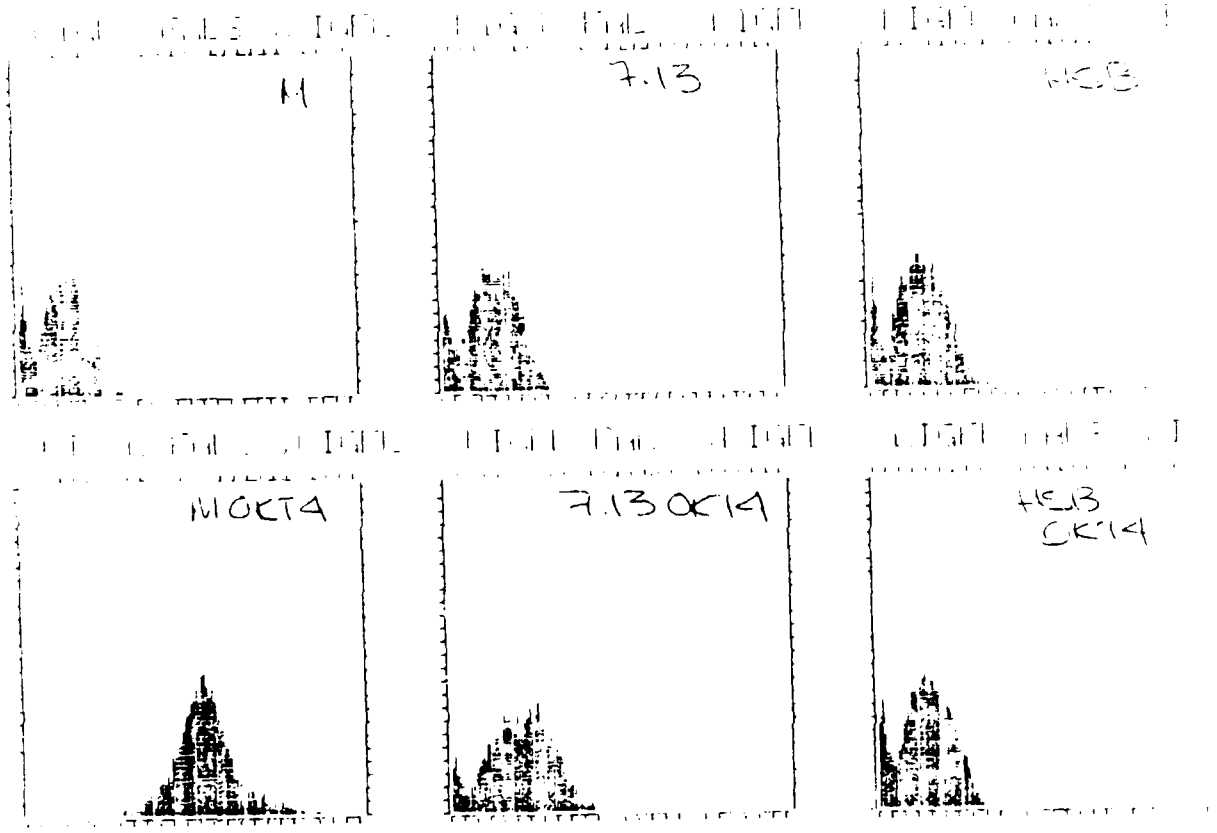
Figure 3. Infection of murine T cell lines: G3CD4.33 (which modulates CD4) in infectable whereas 16CD4.9 (does not modulate) is not susceptible.

Figure 4. A human CD4- CD8- line stains with anti-δ but not $\alpha\beta$.

Figure 5. Immunoprecipitation of a human CD4- CD8- line reveals the $\gamma\delta$ receptor.

Figure 6. The CD4- CD8- line responds to heat shock protein.

FIGURE 1



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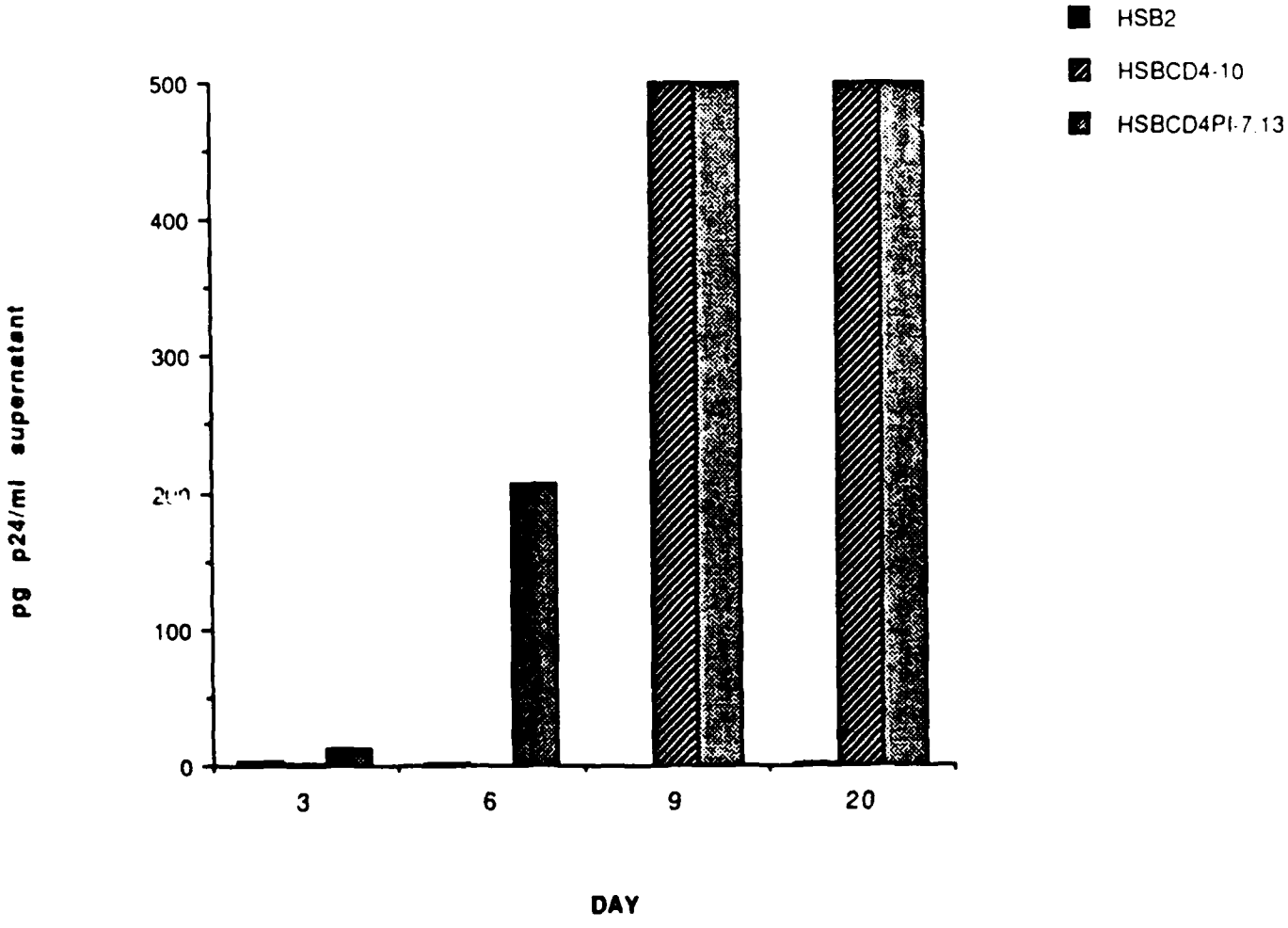
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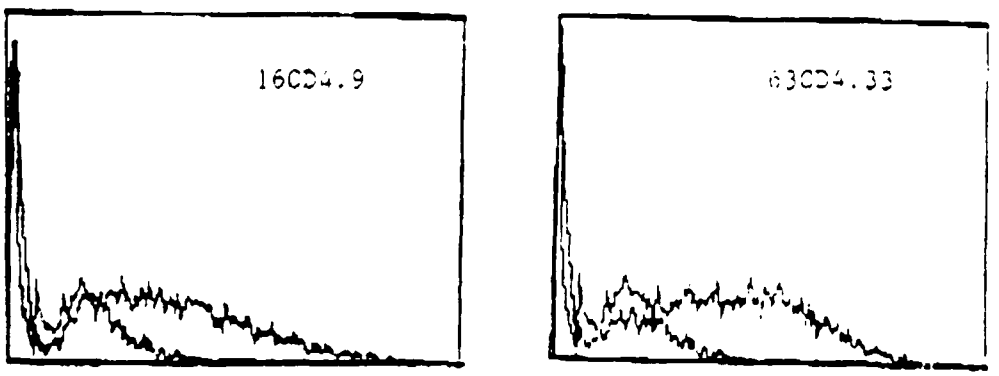
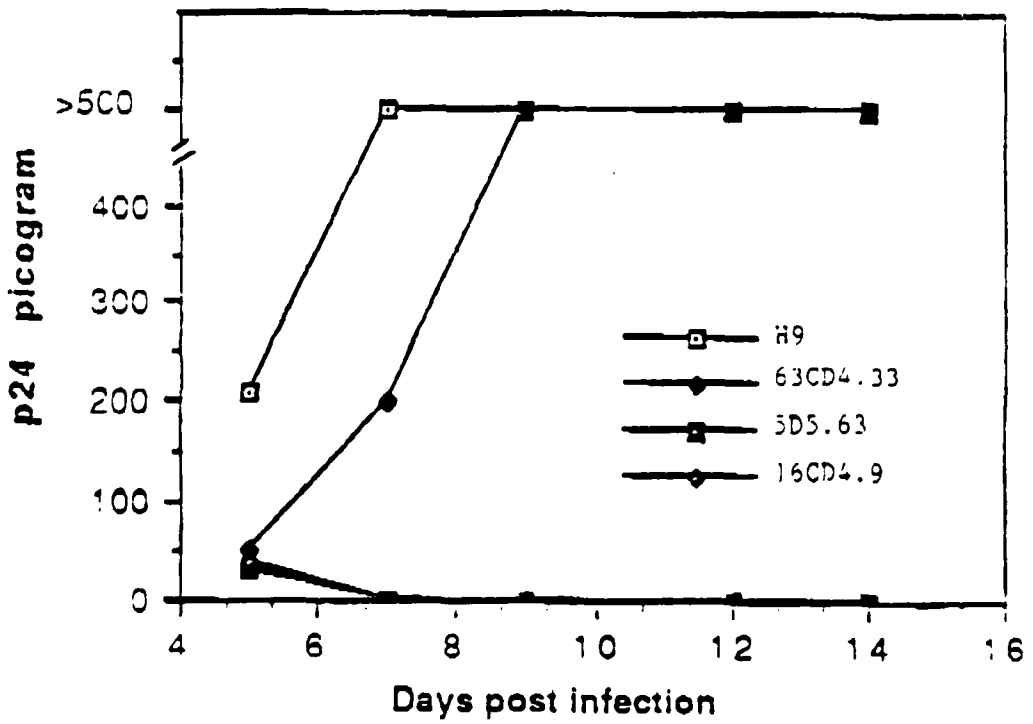
FIGURE 3

Infection of murine T cell hybridoma 63CD4.33 with HIV-1

(a). Expression of human CD-4 proteins on murine T cell hybridomas.



(b). Infection of H9 and 63CD4.33 with HIV-1.



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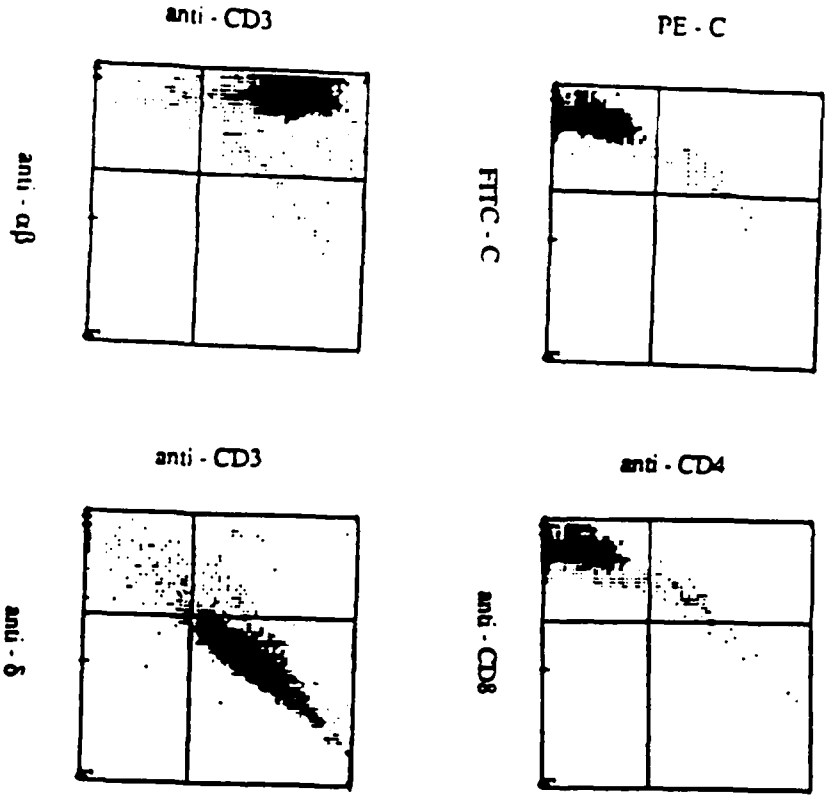

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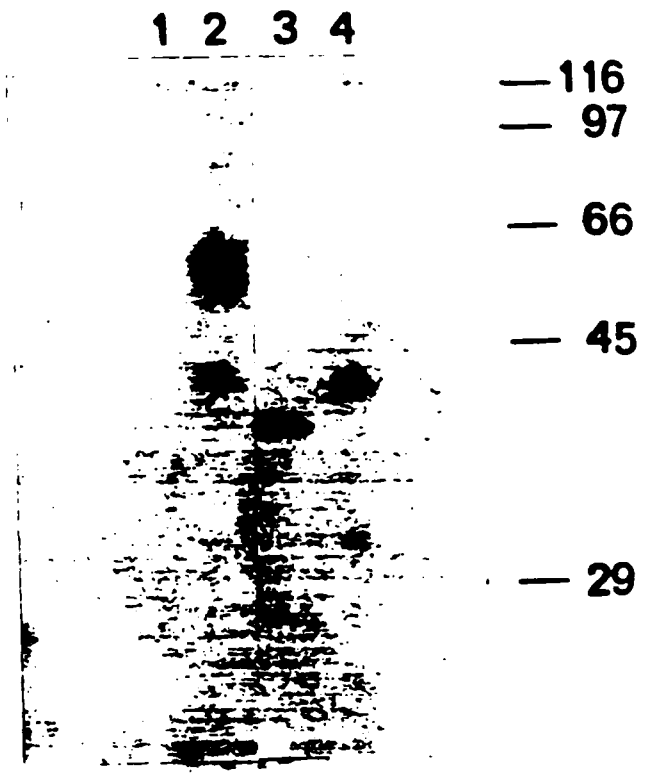

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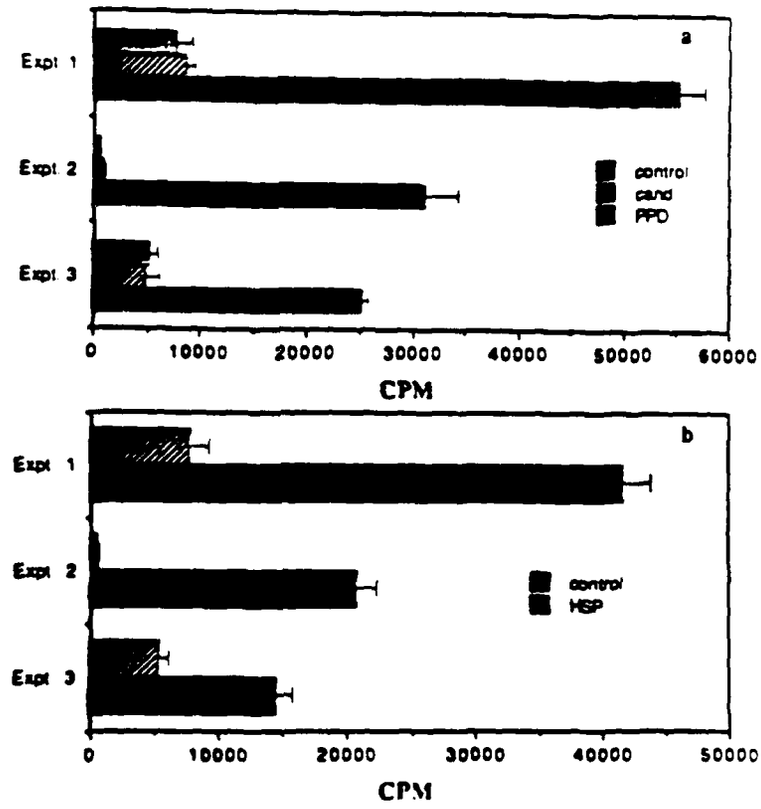
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